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THE ARC-TEST : A STANDARDIZED SHORT-TERM ROUTINE TOXICITY TEST WITH ARTEMIA NAUPLII. METHODOLOGY AND EVALUATION

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ABSTRACT

Considering the need for reliable standardized routine toxicity tests for the marine environment, a short-term bioassay with Artemia nauplii has been developed for routine testing.

The major reason for the selection of brine shrimp as test species is the continuous availability of Artemia under the form of dry cysts from which the larvae are hatched very easily. This unique advantage solves one of the major problems of aquatic ecotoxicological tests, namely stock recruitment and/or culturing ; the Artemia test can be performed anywhere and at any time from dry biological material available "on the shelf".

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An extensive study has been performed on various brine shrimp tests published in scientific literature, the outcome of which has been the development of a simple and inexpensive bioassay suitable as a first screening test for the toxicity ranking of chemicals and as yardstick test for marine biota.

The "Artemia Reference Center"-test (ARC-test) is a short-term, acute bioassay based on the determination of the 24 h LC50 of instar II-III nauplii of a specific Artemia strain. The test procedure has been the subject of two international round robin excercises, one in Europe (sponsored by the Commission of the European Communities), the second in North America, to determine the degree of standardization of the experimental protocol proposed. From the results of these ring tests, in which 59 laboratories participated in the first exercise and eight in the second, onecan conclude that, although the test was entirely new to two thirds of the participating laboratories its repeatability and reproducibility are at least equal to that of the short-term Daphnia test.

KEYWORDS

Marine ecotoxicology, Methods, Bioassays, Artemia, Standardization.

INTRODUCTION

In the general framework of the protection of the marine environment simple and reliable ecotoxicological test methods are necessary to determine the potential impact of xenobiotic substances on the marine biota. Furthermore, correct implementation of the regulatory measures taken at the national as well as the international level requires that the toxicological methods used be both reliable and reproducible.

Although the need for simple standardized routine tests for the marine environment is as high as in freshwater ecotoxicology, it appears that less attention has been paid to developing such tests with marine organisms than with freshwater species.

For practical reasons species with a cryptobiotic stage during their life cycle are most suited for the development of a standard bioassay. The permanent availability of resting eggs from which larvae can be obtained means that :

- there is no need for maintenance of live stock ;
- the tests can be carried out wherever and whenever needed ;
- a sufficient number of test organisms of the same age and physiological condition is always available.

As the brine shrimp Artemia is the only animal species for which cryptobiotic stages (the cysts) are commercially and continuously available all over the world to-date (as a source of live food for fish and crustacean aquaculture), it was chosen as the basis of a simple, inexpensive and reliable short-term test. Here three steps are reported which led to the ARC test.

RESEARCH ON TEST CRITERIA AND METHODOLOGY

Firstly, a thorough review was made of the existing literature dealing with the use of Artemia in experimental pollution research including data on the factors which influence hatching and molting of brine shrimp (Sorgeloos, 1980 ; Vanhaecke et al., 1980). On this basis a list of facts and criteria was drafted which might be taken into consideration for the development of a standard Artemia test :

- the early larval stages of Artemia, which can survive for a few days without feeding, are well suited for acute toxicity testing.
- the nauplii have to be hatched out under strictly controlled conditions of temperature, salinity, aeration, light, and pH ;
- the larvae must be of exactly the same age at the start of every test;
- during the test the larvae may not molt into an instar with a different sensitivity ;
- the tests have to be carried out with cysts of the same geographical origin ;
- the experimental conditions of the test must be defined with great accuracy ;
- a control test with a reference toxicant chemical must be carried out each time in parallel to check both the sensitivity of the larvae and the conformity with the standard procedure.

Starting from these prerequisites a tentative test protocol was worked out and a number of experiments were performed to check the impact of several factors on the practicality of the test procedure, the sensitivity of the test, and the reproducibility of the results, in order to arrive at a simple short-term test with an acceptable reliability (Vanhaecke et al., 1980). The following factors were studies in detail :

- the selection of the instar stage to be used ;
- the duration of the test ;
- the sensitivity of early versus late hatching nauplii ;
- the influence of the exact age of instar II-III nauplii at the start of the test ;
- the influence of the storage conditions of the cysts ;
- the sensitivity of different geographical strains and batches of Artemia ;
- the selection of a standard reference toxicant ;
- the determination of the accuracy and repeatability of the test.

Finally, a 24 h toxicity test with nauplii of a well determined geographical origin, which are all in the instar II-III stage at the start of the test, was found to give the best results with regard to sensitivity, practicality, and reproducibility.

DEVELOPMENT OF THE TEST PROTOCOL

A protocol has been worked out for a short-term toxicity test with Artemia nauplii (Vanhaecke et al., 1981) similar to the protocols of the International Standardization Organization (ISO) for the acute toxicity test on Daphnia and Brachydanio. The principle of the test, as mentioned above, consists of the determination of the concentration which kills 50 % of the Artemia nauplii within 24 h under a set of precisely defined conditions.

The protocol proposed was submitted to and has been discussed in detail during a special workshop on toxicity tests with brine shrimp, at the International Symposium on the Brine Shrimp Artemia salina held in Corpus Christi, Texas, in August 1979 (Persoone and D'Agostino, 1980). At the end of this convention a recommendation was formulated by the experts present "that the reliability and reproducibility of the Artemia test proposed should be evaluated in an intercalibration exercise in order to determine the degree of standardization achieved."

ORGANIZATION OF THE INTERCALIBRATION EXERCISES

Two intercalibration exercises were subsequently organized by the Artemia Reference Center, one in America and one in Europe. The former ring test was set up in collaboration with the Toxicity Section of the Freshwater Institute in Winnipeg, Canada ; the latter has been carried out under contract with the Commission of the European Communities.

The implementation of the European round-robin test and the methodology for processing of the data has been reported in detail in Vanhaecke and Persoone (1981). The American intercalibration exercise was performed independently, but completely in parallel to the European test. The results of both intercalibration tests, which were carried out with two chemical compounds (sodium laurylsulphate and potassium dichromate), are summarized in the Tables I and II.

Table I. Statistical treatment of the data for sodium laurylsulphate

	Europe	America
Number of participating laboratories	59	7
Mean 24 h LC50 ($mg.l^{-1}$)	22.52	20.02
Intra-laboratory standard deviation ($mg.l^{-1}$)	3.27	4.00
Intra-laboratory coefficient of variation (%)	14.52	19.96
Inter-laboratory standard deviation ($mg.l^{-1}$)	5.59	5.06
Inter-laboratory coefficient of variation (%)	24.82	25.25
Number of measurements	143	21
Number of laboratories excluded because of deviation from the experimental protocol	2	0
Number of laboratories excluded because of insufficient repeatability	4	0
Number of laboratories excluded because of insufficient reproducibility	0	0

Table II. Statistical treatment of the data for potassium dichromate

	Europe	America
Number of participating laboratories	59	8
Mean 24 h LC50 ($mg.l^{-1}$)	38.87	42.39
Intra-laboratory standard deviation ($mg.l^{-1}$)	6.65	4.38
Intra-laboratory coefficient of variation (%)	14.54	10.33
Inter-laboratory standard deviation ($mg.l^{-1}$)	13.56	7.48
Inter-laboratory coefficient of variation (%)	34.89	18.49
Number of measurements	146	21
Number of laboratories excluded because of deviation from the experimental protocol	2	0
Number of laboratories excluded because of insufficient repeatability	5	1
Number of laboratories excluded because of insufficient reproducibility	0	0

It should be mentioned that the number of participants in the European intercalibration exercise exceeds by far that for the round-robin test in America (only eight laboratories). The explanation for this disparity was a long postal strike in Canada which seriously interfered with mailing of the materials to and receipt of the data from more than 100 laboratories in North America intending to take part in the round-robin testing.

For sodium laurylsulphate both the mean 24 h LC50 and the intra- and inter-laboratory variation are comparable for the two excercises, the variation being somewhat higher for the American test. For potassium dichromate on the other hand the intra- and inter- laboratory variation is lower for the American test. When comparing the intra-laboratory variability of the Artemia round-robin test to that of the accepted standard freshwater tests with Daphnia and Brachydanio, it appears that the repeatability of the Artemia test is at least equal to that of the Daphnia test (Table III). The inter- laboratory variance data illustrate that the Artemia test is more

reproducible than the Daphnia test and approximates in some cases the values obtained for Brachydanio. These variation data are very satisfactory since the Artemia bioassay was new to two thirds of the participating laboratories. A questionnaire issued at the occasion of the round-robin test revealed that a substantial part of the variance was due to the fact that most participants were not familiar with the bioassay (Persoone and Vanhaecke, 1981).

Table III. Comparison of the intra- and inter-laboratory variation (%) of intercalibration excercises with Artemia, Daphnia magna and Brachydanio rerio

	Artemia		Daphnia	Brachydanio
	Europe	America		
Sodium laurylsulphate				
Intra-laboratory variation	14.5	20.5	-	-
Inter-laboratory variation	24.8	25.3	-	-
Potasium dichromate				
Intra-laboratory variation	14.5	10.3	14[1]	7.6 - 9.4[2]
Inter-laboratory variation	34.9	18.5	39[1]	22.6 - 24.7[2]

[1]Cabridenc 1979a
[2]Cabridenc 1979b

With regard to K_2CrO_7 the sensitivity of the Artemia test seems to be intermediate to that of the Daphnia test (24 h LC50 1.42 $mg.l^{-1}$) and the Brachydanio test (301.7 $mg.l^{-1}$). According to the comments of the participants in the round-robin exercise the Artemia test requires little skill and is not very time consuming.

A critical analysis of all comments was made which led to some minor adjustments in the original protocol and the elaboration of a slightly revised version. The present version of the ARC-test, which is now widely accepted and used is added in appendix.

In conclusion it may be underlined that the ARC-test is a bioassay for the marine environment which not only has received much interest, but which has been submitted to a thorough examination of its reliability and repeatability through round-robin tests in which a substantial number of laboratories (approximately 70) have participated. This interest reflects the need for standardization and cross-comparison in marine ecotoxicological testing. The uniqueness of the ARC-test, which requires only inert cysts, together with the satisfactory reliability of the test, makes this bioassay an invaluable tool as a first screening test for the marine environment and as a routine test for gross toxicity ranking of chemicals. The ARC-test of course also has its limitations, e.g. a relatively low sensitivity, which should be acknowledged. Research is now in progress to work out a second test based on a more sensitive criterion than mortality. A broad spectrum of chemicals is presently being submitted to the ARC-test to determine quantitative structure activity relationships (QSAR's) as well as the interspecific sensitivity relationship of Artemia with other aquatic test species.

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APPENDIX

STANDARDIZED SHORT TERM TOXICITY TEST WITH ARTEMIA NAUPLII (ARC-TEST)

EXPERIMENTAL PROTOCOL

SCOPE AND FIELD OF APPLICATION

This standard method developed at the Artemia Reference Center in the Laboratory for Mariculture at the State University of Ghent in Belgium aims at determining the acute toxicity to nauplii of the brine shrimp Artemia of:

- chemical substances ;
- industrial and domestic effluents considered for dumping, or dumped into the marine environment.

PRINCIPLE

Determination of the concentration which kills 50 % of the Artemia nauplii within 24 h under the conditions described in the present standard. This concentration is known as the 24 h LC50.

LABORATORY

The preparation of the test, the storage of the diluent solution, and all stages of the test procedure described below must take place in an atmosphere free from dust and toxic vapors.

MATERIALS

The test organisms

A homogenous population of instar II-III nauplii (1) hatched out from cysts of a well-defined Artemia strain (2) must be used for the test.

1. Strict application of the hatching and harvesting schedule outlined below should give a homogenous instar II-III population ; a rapid check of the larvae under a microscope may, however, be helpful in case of doubt (Fig. 1).

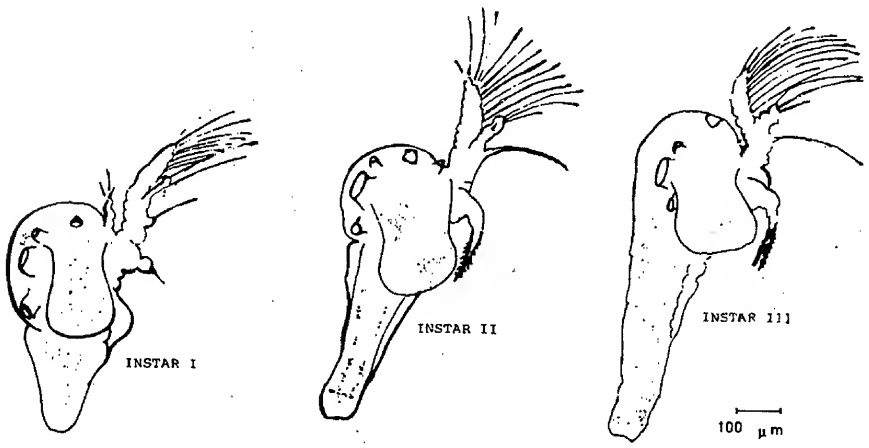

Fig. 1. Morphological characteristics of 1st, 2nd, and 3rd instar nauplii of Artemia salina (after Hentschel, 1968).

2. The Artemia Reference Center has agreed to act as a distribution center of "Reference Artemia Cysts" for fundamental research and for toxicity studies (Sorgeloos, 1980, 1981) It is advised to check cysts of unknown origin with Reference Artemia Cysts to determine if both the tinecourses of hatching and molting, mentioned in this ARC-test, and the sensivity of the larvae for the reference chemical are comparable.

Diluent solution

A standard artificial seawater of 35 ± 1 ‰ is used for the hatching as well as for the test. Whenever possible, the artificial salt mixture of "Instant Ocean® " dissolved in distilled water should be utilized. After aeration and stabilization for 24 h the dilution water should have a pH of 8.0 ± 0.5 and an oxygen content of at least 90 % saturation. If necessary the pH should be adjusted with concentrated hydrochloric acid or sodium

hydroxide. Prior to use, the water should preferably be filtered through 1 µm filter and aerated ; the seawater should not be stored for more th two weeks. Storage at low temperature is recommended.

Laboratory equipment

- constant temperature cabinet : 25 ± 1 °C ;
- glass petri dishes (60 mm x 12 mm) with appropriate covers ;
- Pasteur pipettes with smoothed openings ;
- cylindrical (graduated cylinders) or preferably cylindroconical hatch tubes (diameter ± 35 mm) with a content of at least 100 ml ;
- dissolved oxygen meter ;
- binocular dissecting microscope ;
- small airpump (aquarium pump) ;
- bulb or light tube ;
- usual laboratory materials.

Reference toxicant

The selected reference chemical is sodium laurylsulphate (grade 102 %) This compound is commonly used in surface tension research.

PROCEDURE

Hatching and preparation of the nauplii

For each test approximately 100 mg of cysts are incubated in 100 seawater in a cylindroconical tube or graduated cylinder, at a tempera of 25 ± 1 °C and with lateral illumination by a bulb or light (intensity of at least 500 lux). All the cysts and the hatching nau should be kept in continuous suspension by gentle aeration from a small tube extending to the bottom of the hatching device.

After 18 up to 24 h the aeration is stopped and the nauplii w aggregate at the bottom of the tube are sucked out by pipetting transferred into an Erlenmeyer flask containing 200 ml of seawater. suspension should be aerated gently and kept for exactly 24 h temperature of 25 ± 1 °C and at an illumination of 500 - 1000 lux. Du that time all nauplii will molt to the instar II and some of them eve

the instar III stage (Fig. 1). An aliquot of the nauplii is then poured into a petri dish for subsequent manual distribution to the test petri dishes.

The toxicity test : general

The test is carried out in small glass petri dishes (diameter ± 5 cm). Ten nauplii are transferred with a Pasteur pipette into each dish. The volume of seawater carried over with the nauplii should not exceed 0.05 ml. Ten ml of the respective concentrations of the toxicant (already acclimated at 25 °C), is added to the dishes which are there and incubated in darkness at a temperature of 25 ± 1 °C.

After 24 h the number of dead larvae in each petri dish is determined under a dissecting microscope. The nauplii are considered dead if no movement of the appendages is observed within 10 s. Immediately after counting, the oxygen concentration is measured in the petri dish with the lowest concentration of toxicant that induced a 100 % mortality.

Preliminary test

This test is performed to determine the "critical range". A series of geometrically spaced concentrations or dilutions of the toxicant are prepared with artificial seawater.

Example for chemical substances :
10 000, 1 000, 100, 10, 0.1, 0.01 $mg.l^{-1}$
Example for effluents :
100, 10, 1, 0.1, 0.001 %

The preliminary test is conducted with only one petri dish per concentration. An additional dish with ten nauplii in 10 ml artificial seawater is included as control.

Definitive test

The test aims at the determination of the 24 h LC50, departing from the critical range concentrations determined in the preliminary test. Concentrations (or dilutions) are chosen from a logarithmic scale (Doudoroff et al., 1951). In principle five concentrations should be sufficient. For a satisfactory LC50, however, at least two datapoints must be situated in the

5 - 95 % mortality range. If this is not the case, the test should be repeated with additional intermediate concentrations from the dilution scale. For each concentration (including the control) three replicates are set up.

Checking of the sensitivity of the Artemia nauplii and of the conformity with the experimental procedure

The 24 h LC50, of the reference chemical sodium laurylsulphate must be determined each time in parallel with the definitive test in order to standardize the experimental procedure. The following concentrations of sodium laurylsulphate should be tested in three replicates : 10, 13.5, 18, 24 and 32 $mg.l^{-1}$. To prepare the 100 $mg.l^{-1}$ sodium laurylsulphate stock solution, dissolve the chemical at 25 °C using a magnetic stirrer, since the compound does not dissolve quickly. The stock solution should not be stored for more than 48 h.

CALCULATION AND VALIDITY OF THE RESULTS

The 24 h LC50 can be calculated by graphical interpolation. The percentages mortality between 5 and 95 % are calculated from the average number of dead nauplii per concentration, and plotted on log-probit paper. A straight line is drawn through the points. The intersection of this line with the 50 % mortality abseissa determines the 24 h LC50. An alternative (more precise) procedure is to use the method of Litchfield and Wilcoxon (1949) with which the 95 % confidence limits can be calculated.

The test can be considered valid if the following conditions are fulfilled :

- the percentage mortality in the control does not exceed 10 % ;
- the 24 h LC50 of sodium laurylsulphate is situated between 13.3 and 19.9 $mg.l^{-1}$;
- the dissolved oxygen concentration at the end of the experiment is higher than 2 $mg.l^{-1}$ in the lowest concentration with 100 % mortality of the larvae.

REPORTING OF THE RESULTS

The following data shall always be reported :

- the origin of the Artemia strain and, if possible, the batch number of the commercial brand used ;
- the calculated 24 h LC50, if possible with the 95 % confidence limits ;
- the critical range (0 - 100 % mortality) ;

- the data confirming the validity of the results :
  a) 24 h LC50 of sodium laurylsulphate ;
  b) the percentage mortality in the controls ;
  c) the oxygen content at the end of the test in the lowest concentration of toxicant with 100 % mortality of the larvae ;
- the method of calculation used for the determination of the 24 h LC50
- any deviation from the standard procedure and any problem encountered during the test.

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